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LOCATION OF κ-CASEIN IN MILK MICELLES

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SUMMARY

An attempt was made to determine the whereabouts of κ-casein in the milk casein system by locating antibodies to κ-casein through electron microscopy. No evidence could be found for the occurrence of a κ-casein stabilizing "coat" on casein micelles using either purified antibody or ferritin conjugated antibody. κ-Casein was identified at "bridging points" that interconnect clumps of micelles. Repeated centrifugation and resuspension of casein micelles in fresh solvent removes a κ-casein fraction (serum κ-casein) and this treatment progressively retards rennin clotting of natural milk. This removal of serum κ-casein did not disturb the integrity of milk micelle particles. Similar rennin clotting experiments, using varying weight ratio of purified α_{s1}- and κ-casein in the presence of Ca^{2+}, showed that α_{s1}-casein retarded rennin clotting of simulated casein micelles. These observations have led to the proposal of a new model for casein interaction that is compatible with known micelle characteristics. This model places the large κ-casein aggregate in two different locations in the milk system: (1) 30% is in the free serum phase associated with small amounts of α_{s1}- and β-casein. This rennin-accessible κ-casein is responsible for clot formation and (2) the remaining portion of the κ-casein acts as a nucleating agent for the Ca^{2+}-sensitive caseins. Calcium phosphate salt linkages would give the natural micelle the observed stability required for maintaining structure during heating, concentration and dilution.

INTRODUCTION

Phosphoproteins comprise an unusual complex group of molecules making up approx. 80% of the protein in milk. Studies of the optical rotatory dispersion and viscosity of the individual casein species have shown their structures to be essentially unordered[1,2]. The caseins tend to undergo a high degree of ionic strength dependent hydrophobic aggregation[2–4]; however, in spite of (or because of) these properties, they

Abbreviation: γG_{AK}, isolated γ-globulin with anti-κ-casein activity.

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occur in milk as large spherical colloids, usually referred to as casein micelles*, which show remarkable stability against the effects of temperature, dilution and concentration. Numerous studies have been made on the mode of interaction of the individual proteins which result in the stable suspension that occurs in milk.

The presence of κ-casein in the micelles has long been recognized as being one of the most important factors responsible for stabilizing α_{s1}-and β-casein in the presence of Ca^{2+} (ref. 5). Several models have been proposed to explain the interaction of κ-casein with the other caseins. The theory of NOBLE AND WAUGH[6] (see also ref. 7 for a complete review of micelle models) suggests that α_{s1}-casein, through hydrophobic interaction, forms a core, with low molecular weight units of κ-casein covering the surface like a coat. This model does satisfy the requirement of availability of the κ-casein to hydrolysis by rennin and can explain micellar stability during close approach and dilution. However, the model cannot explain the ability of β-casein to move so readily into the serum phase upon decreasing the temperature to 4° (as if no coat existed) nor has the important role of colloidal calcium phosphate in micellar stability[8] been accounted for.

The purpose of the present investigation was to locate κ-casein in milk micelles by electron microscopy, using micelles reacted with ferritin labeled antibodies to κ-casein, and examination of the rennin clotting rates of simulated and natural micelles.

MATERIALS AND METHODS

Casein preparation

κ-Casein (genetic type BB) was isolated by the procedure of MCKENZIE AND WAKE[9] as modified by M. P. THOMPSON (personal communication). Briefly, the method involves preparation of Ca^{2+}-free Fraction S casein according to the method of WAUGH AND VON HIPPEL[5]; this was then precipitated twice with 1 M ammonium acetate in 50% ethanol. Precipitates were dissolved in a large volume of 0.005 M NaCl (instead of urea), followed by dialysis against the same solvent. The κ-casein obtained was of high purity according to polyacrylamide gel electrophoresis[10] (4.5 M urea, Tris–EDTA–borate buffer, pH 9.0) with added mercaptoethanol (1%). α_{s1}-Casein (genetic type BB) was prepared by the method of ZITTLE AND CUSTER[11].

Antibody preparation

Antiserum to κ-casein was prepared by injecting rabbits with 50 mg of κ-casein in complete Freund's adjuvant. After 3 weeks, a second injection of 50 mg was administered with incomplete Freund's adjuvant. The rabbits were bled 3 weeks later, and sera were collected and stored at −10°. The sera gave varying titers to κ-casein, ranging from 0.4 to 0.85 mg of κ-casein precipitated per ml of serum, as determined by the Folin–Ciocalteu method as modified by JOLLÈS[12].

The γ-globulin fraction possessing anti-κ-casein activity (γG_{AK}) was separated from the antiserum by precipitation with 40% $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose according to the method of LEVY AND SOBER[13]. It was approx. 95% pure according to immunoelectrophoresis[14] against antiserum to whole rabbit serum.

* The term micelle used in this sense should not be confused with the classical soap micelle to which it bears no resemblance in charge, size or shape.

Conjugation of γG_{AK} with ferritin

Equine ferritin (6 times crystallized, Cd^{2+}-free, Calbiochem*) was conjugated to the antibody with bis-diazotized benzidine according to the method of GREGORY AND WILLIAMS[15]. The uncomplexed globulins were removed by gel permeation on Sephadex G-200 in 0.15 M NaCl–0.05 M cacodylate buffer (pH 7.0) at 1°. No attempt was made at this point to remove free ferritin from the γG_{AK}–ferritin complex, since it was found that unbound ferritin could easily be washed from the sample.

Electron microscopy

Milk micelles were prepared for the electron microscope by the method of CARROLL *et al.*[16] using 1% glutaraldehyde as the fixative. Micrographs were taken with an RCA EMU 3-G microscope using a 25-μ aperture at 100 kV acceleration voltage.

Sialic acid

Sialic acid was determined by the method of WARREN[17] using the alkaline hydrolysis treatment recommended for glycoproteins.

RESULTS

Fig. 1 is an electron micrograph of casein micelles from skim milk. This picture shows a "normal" distribution of micelle sizes (500–2500 Å) with the average size around 1300 Å. It is interesting to note that the small background particles in Fig. 1 are not sedimentable by centrifugation at 100 000 × g for 60 min (Fig. 2), while all large micelles can be removed. These small particles are 200–250 Å in diameter.

κ-Casein, isolated by the modified MCKENZIE AND WAKE[9] procedure, is shown in Fig. 3. The individual κ-casein particles measure 185–200 Å, and some clumping is evident in this micrograph.

κ-Casein prepared by other methods, *e.g.* the urea–sulfuric acid method[11], closely resembles that seen here. The overall shape similarity of the small background particles in Figs. 1–3 should be noted.

Initial attempts to observe antibody interaction with κ-casein were made with the unlabeled γG_{AK}. Equal weights of γG_{AK} and κ-casein were mixed in 0.15 M NaCl–0.05 M cacodylate buffer (pH 7.0) (10 g/l total protein) and allowed to stand 2 h at 37°, then overnight at 1°. The precipitate was removed by low speed centrifugation and the supernatant applied to Sephadex G-200. Gel-filtration resulted in two eluted components corresponding to the γG_{AK}–κ-casein complex (at V_0) and the retarded γG_{AK} peak.

In order to assess whether the antibody combining site(s) would inhibit or compete with α_{s1}-casein binding to κ-casein, the ability of the soluble complex to stabilize α_{s1}-casein against precipitation by Ca^{2+} was determined. Comparison of the stabilization effects of κ-casein and the complex was made according to the method of ZITTLE[18]. The γG_{AK}–κ-casein complex had one-third the stabilizing effect upon α_{s1}-casein (w/w) as κ-casein alone. The purified γG_{AK} did not stabilize α_{s1}-casein

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

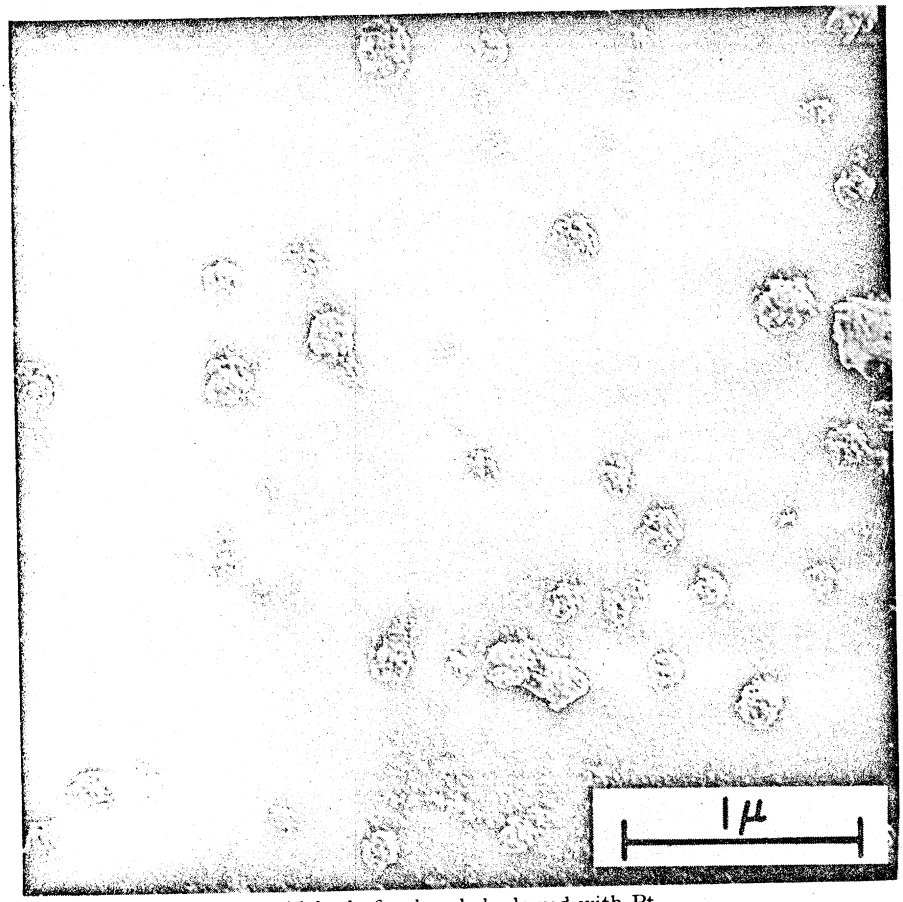

Fig. 1. Skim milk, glutaraldehyde fixed and shadowed with Pt.

against precipitation by Ca^{2+}. Determination of the stabilizing ratio on a mole basis cannot be made since the molecular weight of the complex is unknown.

κ-Casein contains approx. 2% sialic acid. This property has proved useful for quantitation of this protein. The average of four sialic acid determinations of κ-casein and the γG_{AK}–κ-casein complex showed they contained 1.81 and 1.24% sialic acid, respectively. The value for γG_{AK}–κ-casein was corrected for the contribution of γG_{AK}, which as a control, gave an analysis of 0.18% sialic acid. This determination shows that about two-thirds of the γG_{AK} complex is κ-casein.

Antibody interaction with natural casein micelles was examined by electron microscopy. The amorphous structure of these micelles, however, did not allow clear definition of the anti-κ-casein combined to the micelle. Repeated attempts to locate the antibody, either through changes in electron density or through identification of the characteristic gamma globulin structure, were unsuccessful. Trials with a variety of sample preparative techniques gave no evidence of a surface layer of γG_{AK} surrounding the micelle as would be anticipated from the NOBLE AND WAUGH model[6].

Attempts were then made to use the ferritin–antibody conjugate to locate κ-casein on natural micelles. 5 ml of fresh skim milk was fixed with glutaraldehyde

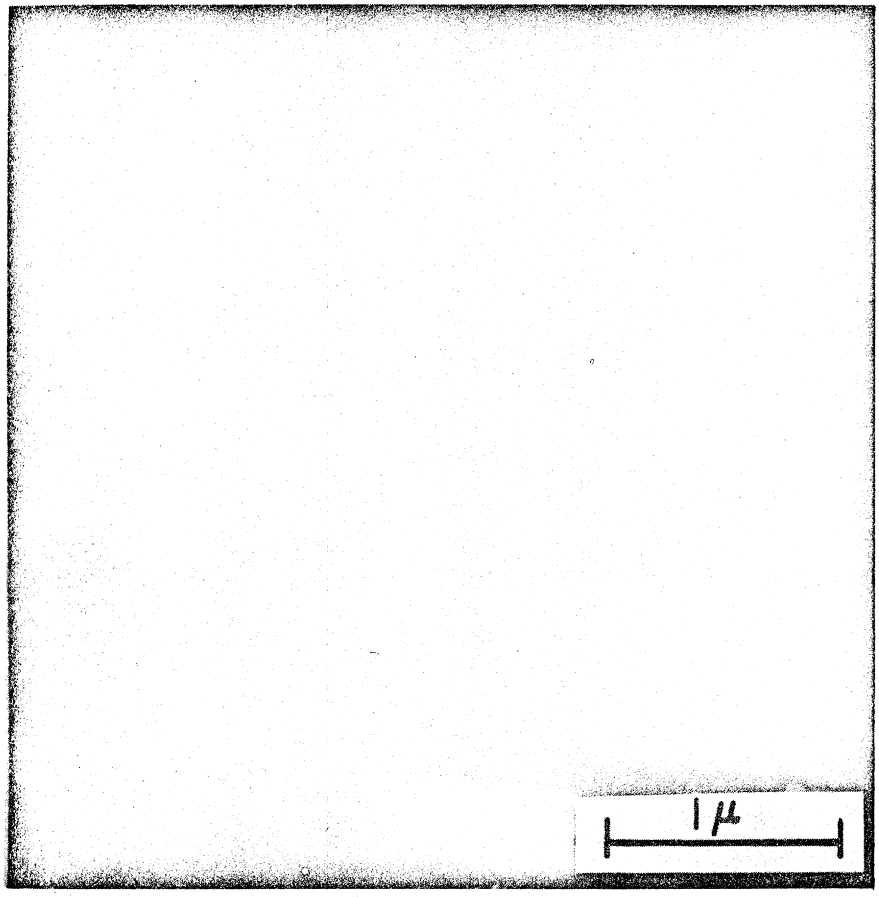

Fig. 2. The supernatant fraction resulting from centrifuging skim milk (Fig. 1) at 100 000 × *g* for 60 min, glutaraldehyde fixed and shadowed with Pt.

and the fixative removed by sedimentation of the micelles at 100 000 × *g* and 20°. The pellet of casein micelles was dispersed in 10 ml of 0.15 M NaCl–0.05 M cacodylate buffer (pH 6.6) again centrifuged and resuspended.

2 ml of approx. 0.1% solution of the gel-filtered ferritin–antibody conjugate was added to 2 ml of the fixed micelle suspension. After standing with occasional agitation at 25° for 1 h, the mixture was centrifuged and resuspended twice as before. The labeled micelles were then deposited on grids and examined without addition of a stain. The results can be seen in Fig. 4. Immunodiffusion experiments have shown that glutaraldehyde fixation of either purified κ-casein or skim milk does not inhibit antibody combination (*cf.* ref. 19). This procedure using prefixed micelles was found superior to the addition of conjugate to unfixed skim milk since subsequent removal of free ferritin or unbound ferritin conjugate was facilitated.

Fig. 4 shows that localized deposits of ferritin appear in certain areas surrounding the micelles (ferritin appears as the black dots). The micelles, however, which appear smooth and grey due to lack of a stain contrast, do not show any ferritin on their surface, but the label appears at bridging points between micelles. The bridging ob-

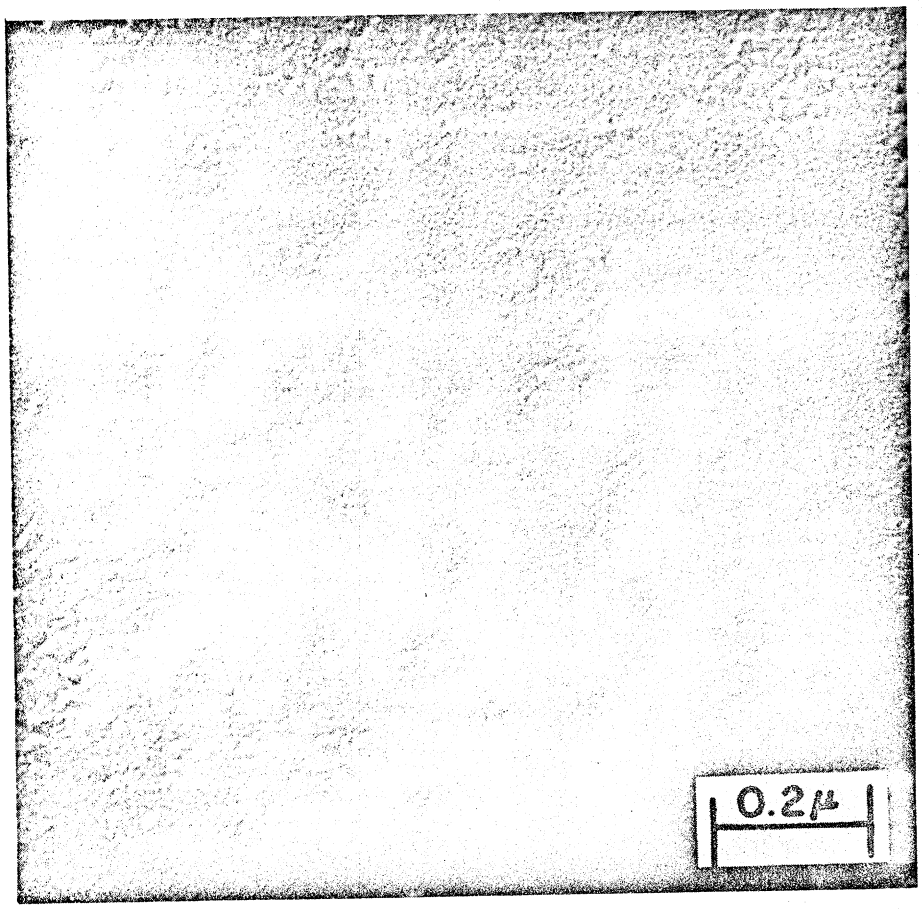

Fig. 3. Purified κ-casein glutaraldehyde fixed and shadowed with Pt.

served here may be an artifact introduced by the centrifugal packing of the micelles, although similar effects have been observed in evaporated milk gels and rennin clots of milk. No free ferritin was found in the electron micrographs of duplicate experiments. Control experiments replacing the antibody conjugate with ferritin itself did not show ferritin binding. Pretreatment of skim milk with γG_{AK} to block the combining sites prior to exposure to the antibody-ferritin conjugate showed that no additional conjugate binding to the micelles occurred.

Experiments were performed to identify the background material seen in electron micrographs of skim milk (Fig. 1) which seems to resemble purified κ-casein (Fig. 3) in size and shape. Fresh whole milk was skimmed by centrifugation for 10 min at 5000 × *g* and 25°. The skim milk was transferred to 40-ml centrifuge tubes and spun at 100 000 × *g* at 4° for 60 min. The supernatant containing the nonsedimentable protein (Fig. 1) was decanted, and the pellet was resuspended to the initial volume with JENNESS–KOOPS[20] buffer. Individual samples were dispersed by finely dividing the pellet with a spatula and agitating the capped tube 15 min. The procedure was repeated for a total of 6 extractions. Samples were set aside after each centrifugation by removing

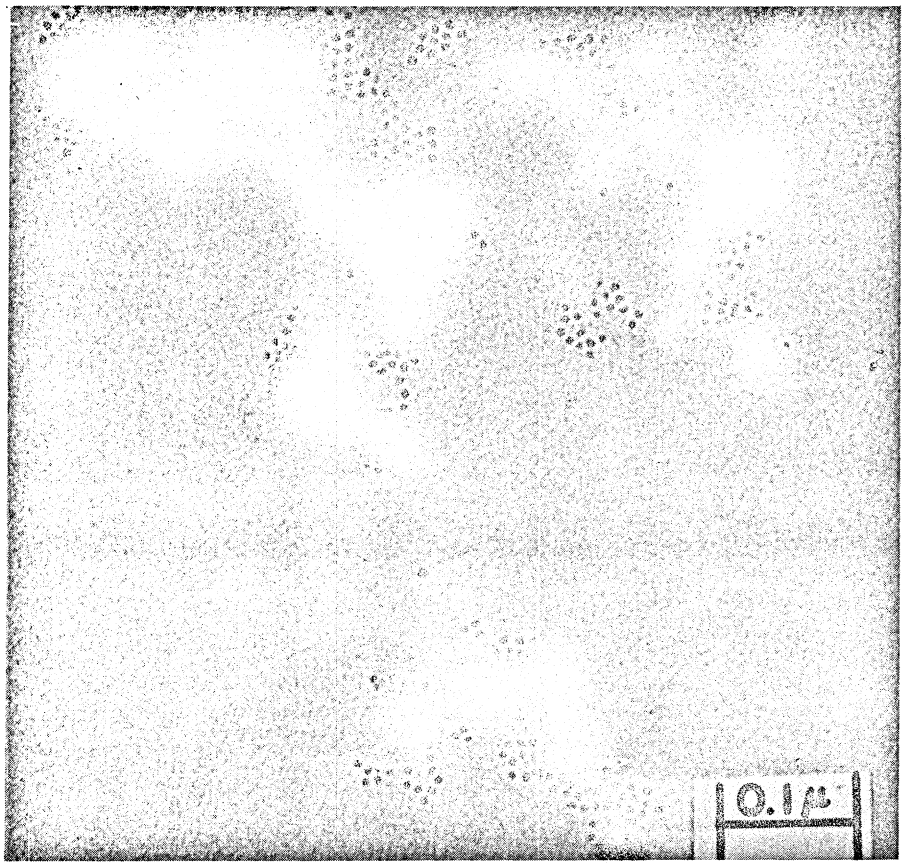

Fig. 4. Glutaraldehyde fixed skim milk plus the conjugated ferritin–γG_{AK} complex. This mixture was reacted 60 min at 25°, fixed with glutaraldehyde, washed twice by centrifugation and photographed without shadowing or staining.

2 tubes from the rotor, one for measurement of clotting time and the other for sialic acid determination. All samples, supernatant and pellet, were dialyzed exhaustively against water and lyophilized prior to sialic acid analyses. The clotting time experiments were done by dispersing the pellets for 3 h at 37°. After this time, 0.2 ml of a 0.1% rennin solution (EC 3.4.4.3) was added to each sample. Fig. 5 shows the time required for clotting, together with the concentration of sialic acid (μmoles/mg protein) in the supernatant and the pellet after each centrifugation.

The increase in the renning clotting time noted for each successive micelle "washing" indicates progressive removal of κ-casein. This conclusion is substantiated by the reduction of sialic acid in the pellets; the sialic acid being lost to the serum phase. Visual comparison of the type of rennin clot formed showed that the samples given more washings produced less firm clots. For example, the skim milk sample (0 in Fig. 5) gave a firm clot capable of syneresis, while No. 6 (6 times washed) formed loose clumps, but no continuous gel, even after standing several hours.

The resuspended protein after 6 washings still appeared as opalescent as skim

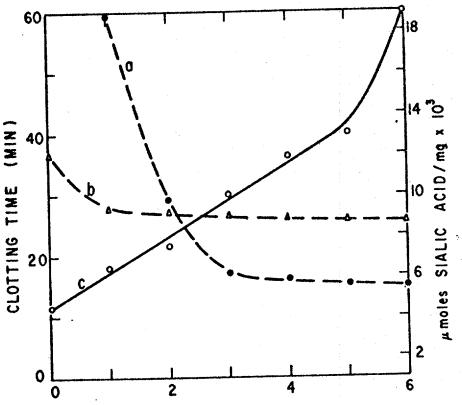

Fig. 5. Effect of repeated centrifugation (100 000 × *g* for 60 min) and resuspension (in milk–salts-stimulating buffer) on the sialic acid concentration and rennin clotting time of a skim milk suspension. (a) Sialic acid concentration (μmoles/mg of dialyzed and lyophilized protein) of the supernatant fraction resulting from each centrifugation; (b) sialic acid concentration of the pellet; and (c) rennin clotting time of the protein suspensions at 37°.

milk, and no change in the size or shape of the micelles was found when they were examined by the electron microscope. Polyacrylamide gel electrophoresis in urea–mercaptoethanol showed no major compositional changes in the pellets after 6 washings. The electrophoretic patterns of the supernatants, however, always show κ- and β-casein as well as some α_{s1}-casein. Whey proteins decrease rapidly after the first treatment. The presence of β-casein is not unexpected in the supernatants as it has been shown to slowly dissolve into the serum phase at 4° (see ref. 20). Electrophoresis showed, qualitatively, that the proportion of β-casein to κ-casein increased with further washing, although total protein decreased.

As a further test to demonstrate the importance of serum κ-casein to the rennin clotting reaction of micelles, the effect of rennin on simulated micelles prepared from purified casein components was studied. Various weight ratios of α_{s1}-casein to κ-casein were mixed and dialyzed against Ca^{2+}-containing buffers (0.15 M NaCl–0.01 M imida-

TABLE I

RENNIN CLOTTING TIME OF VARIOUS WEIGHT RATIOS OF α_{s1}- AND κ-CASEIN IN THE PRESENCE OF Ca^{2+}

Sample	*Original weight ratio α_{s1}-casein to κ-casein*	*κ-Casein (mg/ 4 ml)*	*α_{s1}-Casein (mg/ 4 ml)*	*Time to form clot (min)*
1	—	4	0	6
2	3:1	4	12	65
3	5:1	4	20	125
4	10:1	4	40	215
5	20:1	4	80	435
6	30:1	4	120	—*

* No visible evidence of clotting even after 24 h.

zole · HCl–0.02 M $CaCl_2$, pH 6.7) at 37° for 4 h with four changes of buffer and constant stirring. The addition of Ca^{2+} to the mixed casein proteins causes formation of aggregates which somewhat resemble milk micelles in several of their properties, such as appearance, clotting behavior and sedimentability. This suspension of simulated or "synthetic" micelles was centrifuged at 1500 × *g* for 10 min to remove any insoluble α_{s1}-casein–Ca^{2+} complex, and the supernatant protein was determined by $A_{290\ m\mu}$ readings on aliquots adjusted to pH 12. The simulated micelle samples were then adjusted to equal protein concentration (2.2 mg/ml) using the afore-mentioned buffer warmed to 37°, and 0.1 ml of a 0.1% rennin solution added to a 3-ml sample. The clotting times of the samples were then measured. The results are reported in Table I. The protein weights reported are the initial concentrations prior to exposure to Ca^{2+}. Total stabilization of α_{s1}-casein was achieved with Samples 2, 3 and 4 based on $A_{290\ m\mu}$ readings. At higher weight ratios (Samples 5 and 6), κ-casein did not effectively stabilize all the α_{s1}-casein present; however, the total protein in suspension did increase over that observed in Sample 4. It is generally assumed[18] that all κ-casein remains in solution under such conditions and that the precipitate which results from Ca^{2+} addition is pure α_{s1}-casein.

As was also observed in the rennin clotting reaction of the washed natural skim milk micelles, treatments which diminish the amount of serum κ-casein retard clot formation. Here there is a direct relationship between clotting time and the amount of α_{s1}-casein. Similar effects were found in other experiments where changes were made in ionic strength, Ca^{2+} and protein concentration.

DISCUSSION

The various proposals which have been presented on casein micelle structure have all assumed that the κ-casein interaction with α_{s1}-casein proceeds with the former dissociating into lower molecular weight units prior to combination. No chemical evidence has ever demonstrated such dissociation of κ-casein in the absence of dissociating solvents; however, physical experiments using sedimentation data have suggested it[6]. Recent studies[22] comparing gel permeation chromatography on Sephadex G-200 to ultracentrifugation indicate no reduction in molecular size of the α_{s1}-casein–κ-casein complex, but rather an increase in the size of the κ-casein aggregate. Therefore a thorough reexamination is underway to explain the apparent discrepancy between the two techniques.

κ-Casein is unique among the caseins in that it contains 2 half-cystines which appear to enter into intermolecular crosslinking[23,24]. In addition, strong hydrophobic interactions can be shown. These covalent and nonpolar forces yield a protein which characteristically is eluted at the void volume on Sephadex G-200[25], gives a sedimentation coefficient of 14 S (see ref. 6) and will not penetrate 6% polyacrylamide gels unless urea and mercaptoethanol are present[23]. Aqueous salt solutions of κ-casein are often opalescent, and electron microscopy shows large aggregates, 185–200 Å in diameter (Fig. 3). The ensuing discussion of our micellar model will be based, therefore, upon the premise that the stabilization effect of κ-casein on α_{s1}- and β-casein results primarily from a high-molecular-weight aggregate form of κ-casein.

It seems unlikely that a blockage of the κ-casein–γG_{AK} interaction site through κ-casein–α_{s1}-casein binding is occurring. This was tested indirectly by examining the

κ-casein–γG_{AK} stabilizing ability on calcium α_{s1}-caseinate. It was found that the antibody–κ-casein complex gave 50% (w/w) of the stabilizing effect of κ-casein alone. Furthermore, immunodiffusion tests on skim milk (where κ- and α_{s1}-casein are free to interact) and isolated κ-casein show no difference in antigenic response.

Tagging the anti-κ-casein antibodies with electron-dense ferritin indicated no localization of κ-casein on the micelle surface. It did give evidence that κ-casein must be a component of the bridging network which interconnects the micelles. Several attempts were made to use ultrathin sections of micelles to find out if the κ-casein is located in the micelle interior. It was found, however, that plastic embedding media (Epon, methacrylate or Vestopal resin) bind considerable amounts of ferritin non-specifically, and it was impossible to remove this ferritin by mild washing treatments of the sections.

The results of the rennin clotting rate studies of washed milk micelles and mixtures of α_{s1}- and κ-casein suggest that the role up to now described for κ-casein in the stabilization of Ca^{2+}-sensitive caseins is inadequate. Repeated sedimentation and resuspension of skim milk micelles shows that κ-casein is being removed in part with each extraction. It is postulated that it is this free serum κ-casein which is responsible for rennin clotting of milk. The free or "rennin accessible" κ-casein does not play any part in micelle stabilization. This was established by the only criteria presently available to demonstrate micelles after removal of this κ-casein: (1) visual identification of an opalescent suspension similar to milk, (2) stability to centrifugation and resuspension and (3) size measurements in the electron microscope. The necessity for washing resuspended casein micelles 6 times to essentially eliminate clot formation, demonstrates that the free κ-casein does weakly associate with the micelles during centrifugation. This occlusion of κ-casein to the micelles (seen as micellar bridging points in the tagged antibody experiments) may, of course, be a result of poor dispersal of the ultracentrifugal pellet of casein. In any event, removal of this material, which is demonstrably involved in clot formation, does not alter the micellar stability.

Examination of the casein pellets by polyacrylamide gel electrophoresis and analysis for sialic acid content showed that κ-casein was still present after 6 washings. Carbohydrate tests showed that 70–75% of the original amount of κ-casein was still an integral part of the micelle. These figures are in agreement with those of ROSE *et al.*[26] for the ratio of micellar to serum κ-casein in milk.

Further evidence that a free κ-casein aggregate is responsible for the formation of the rennin clot is shown by examination of the enzyme's behavior toward simulated micelles (Table I). At increasing α_{s1}-casein to κ-casein weight ratios, rennin-induced clotting of the system became successively less through α_{s1}-casein interaction with free κ-casein. GARNIER *et al.*[27] have similarly shown that α_{s1}-casein inhibits rennin action on κ-casein.

The conclusion that the free serum κ-casein is responsible for rennin clotting and that no surface κ-casein can be shown, leads to a clearly different conception of casein interaction. It is proposed that the 70% micellar κ-casein functions as a nucleating agent for the initial formation of casein micelles. This model requires that a high-molecular-weight κ-casein aggregate be placed in the center of the micelle and be surrounded by the insoluble Ca^{2+}-sensitive casein. Such a nonspecific type of interaction would easily accommodate more than one κ-casein aggregate within the micelle. However, this model would predict such an occurrence to a limited extent, which is

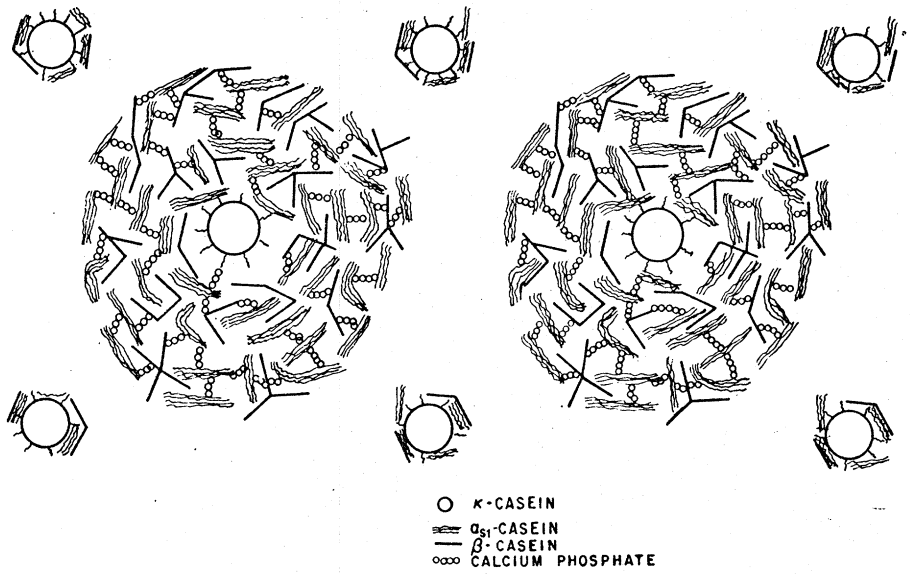

Fig. 6. Proposed model for the location of κ-casein and Ca^{2+}-sensitive proteins in milk micelles (the large protein aggregates) and in the supernatant particles (smaller aggregates).

in agreement with the reported relationship between micelle sedimentation rate and κ-casein content[26,28].

A drawing of this model incorporating α_{s1}-, β-, κ-casein and calcium phosphate is shown in Fig. 6. No attempt has been made to construct the model to scale because size information is not available for these proteins at the normal ionic strength and Ca^{2+} concentration found in milk. Neither is any information available on the state of the calcium phosphate. The larger aggregate is a representation of a micelle. The central κ-casein aggregate is pictured with glycomacropeptide tails on its surface, while the interior is held together by strong disulfide-hydrophobic bonding similar to that described recently by HILL AND WAKE[29]. The κ-casein core is surrounded by aggregates of α_{s1}- and β-casein molecules. The individual α_{s1}-casein molecule is represented as an unordered rod which in turn has hydrophobically associated with several other α_{s1}-caseins to make an aggregate of the order of 100 000–200 000 daltons[4]. β-Casein, on the other hand, is pictured as a random molecule with greater rod-like character which also self-associates in a branched-chain manner[2,30]. Colloidal calcium phosphate forms salt bridges within this network to give the micelle rigidity. Limited movement of proteins and ions into and out of the serum phase does occur; however, the system of salt bridges keeps the micelles stable. The nonequilibrium character of micelles has been shown[21]. In this model size limitation would be dictated by the exhaustion of the soluble protein in the immediate vicinity of the growing micelle.

The smaller protein aggregates shown in Fig. 6 represent free serum κ-casein particles seen in Figs. 1–3. These particles, consisting mainly of κ-casein, which is the same size as the micellar κ-casein, have been shown to associate with α_{s1}- and β-casein in milk serum[26]. The κ-casein component would still be freely accessible to rennin

attack. It then follows that this hydrolysis would cause the highly insoluble para-κ-casein particle to strongly bind to the micelle surface and begin connecting the micelles together, thus forming the clot. This mechanism is compatible with the electron microscopic observations of PETERS AND DIETRICH[31] and additional studies in this laboratory.

A recent report by LAWRENCE AND CREAMER[32] on rennin action on κ-casein in the presence of other caseins showed that α_{s1}- and β-caseins prevent aggregation of para-κ-casein but they do not inhibit formation of para-κ-casein. These experiments, carried out in the absence of Ca^{2+}, indicate that in the primary α_{s1}-casein–κ-casein interaction product α_{s1}-casein is oriented so that it effectively prevents para-κ-casein agglomeration (clotting). This orientation interpreted in terms of this model would be as a surface coat of α_{s1}-casein surrounding the κ-casein aggregate. In any event care should be exercised in interpreting rennin action on caseins so that a careful delineation is made between clot formation and κ-casein hydrolysis to para-κ-casein and glycomacropeptide.

This model can explain numerous observations made with both native and simulated micelles. Movement of β-casein out of the micelle into the serum phase can easily take place if the temperature is lowered. The highly important role of colloidal calcium is incorporated to accommodate reported observations of several workers[8,21,33]. Clot formation can be visualized as a progressive knitting of the micelles into a three-dimensional network mediated through the insoluble serum para-κ-casein. The relationship between micelle size and κ-casein concentration[26,28], *i.e.* the higher the proportion f κ-casein the smaller the micelle, can also be accounted for by this model.

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